Production of Monoclonal Antibodies to *Corynebacterium sepedonicum*

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ABSTRACT

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Five hybridoma cell lines, VRS-H-004-CS3-1 to VRS-H-008-CS3-5, secreting monoclonal antibodies McAb 1 to McAb 5, respectively, to *Corynebacterium sepedonicum* were produced by fusing splenocytes from immunized BALB/c mice with P3/NS1/1-AG4-1 (NS-1) myeloma cells. Both whole cells and a lithium chloride cell extract were used as immunogen and as antigen in enzyme-linked immunoassays for screening hybridomas. Monoclonal antibodies produced by all five hybridomas reacted in indirect immunofluorescence with 19 strains of *C. sepedonicum* from 18 different geographic areas. McAb 3 and 4 reacted with strains of *C. fascians, C. insidiosum, C. michiganense* pv. *iranicum*, and *C. poinsettiae*. McAb 1 reacted weakly with *C. fascians* and *C. insidiosum*. McAb 2 and 5 did not react with any of the ten other plant pathogenic corynebacteria species tested. All the monoclonal antibody preparations except McAb 5 reacted with one or more unidentified nonpathogenic bacteria isolated from potato.

Additional key words: bacterial ring rot.

The difficulty in detecting low populations of *Corynebacterium sepedonicum* (Spieck. and Kotth.) Skapt. and Burkh., the incitant of bacterial ring rot of potato, is a limiting factor in the study of this organism. For example, the determination of inoculum sources and the development of pathogen-free seed potatoes require that the presence of low numbers of pathogenic bacteria be confirmed in the absence of disease. Serological procedures currently are used as sensitive detection methods, but their precision is severely hampered by the lack of specificity in available antisera. Serological cross-reactions of other bacteria with rabbit antisera produced against *C. sepedonicum* have been well documented (1,4,5,7,11).

Recently, the potential specificity of serological procedures has been enhanced with the advent of hybridoma technology (9). This technology provides a means for producing monoclonal antibody preparations in which all immunoglobulin molecules in the preparation have identical specificity for a particular epitope. Thus, if cells of *C. sepedonicum* possess a unique epitope that is not present on other bacteria, it should be possible to produce monoclonal antibodies that are specific only for the ring rot bacterium. Although very little is known about cell wall antigens of *C. sepedonicum*, the specificity of monoclonal antibodies is most attractive for a diagnostic test.

This study was initiated to determine the possibility of developing hybridomas that produce monoclonal antibodies useful for serological detection of *C. sepedonicum*.

MATERIALS AND METHODS

Bacterial strains. *Corynebacterium sepedonicum* strain CS3 was used as antigen for production and screening of monoclonal antibodies and as source of extracted antigen preparations. Other bacterial strains used to test antibody specificity included 18 other strains of *C. sepedonicum* (Table 1) and the following plant pathogenic corynebacteria (6): *C. betae* CB101 and CB103, *C. fascians* CF17 and CF107, *C. flaccumfaciens* CF3 and CF8, *C. insidiosum* CI16 and CI102B, *C. michiganense* pv. *michiganense* CM1 and CM8, *C. michiganense* pv. *iranicum* CI147 and CI148, *C. michiganense* pv. *tritici* CT102 and CT104, *C. oortii* CO101 and CO102, *C. poinsettiae* CP2 and CP109, and *C. rathayi* CR1 and CR101. Also used to test antibody specificity were 13 unidentified bacteria isolated from potato stems and tubers and selected on the basis of cross-reactivity with rabbit antiserum prepared against *C. sepedonicum* (4). All cultures were grown and maintained on a yeast extract-glucose-mineral salts (YGM) medium (7).

Antigen extraction. Cell wall antigens were extracted with LiCl in a procedure similar to that used by Yakrus and Schaad (14) for *Erwinia chrysanthemi*. Cells were grown in YGM broth for 3–4 days, pelleted by centrifugation at 12,000 *g* for 30 min, and resuspended in 20 ml of 0.2 M LiCl/g wet weight of cells. The suspension was stored overnight at 4 C and subsequently agitated for 2–3 hr at 45 C. Cells were removed by centrifugation at 12,000 *g* for 30 min and the supernatant fraction was centrifuged at 100,000 *g* for 2 hr. The final pellet was resuspended in distilled water and used as the cell wall extract.

SDS-PAGE. Cell wall extracts were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 11% vertical slab gels. Protein bands were stained with Coomassie Brilliant Blue R250 (BioRad Laboratories, CA). Carbohydrates were stained with Schiff's reagent (Sigma Chemical Co., MO).

Monoclonal antibody production. BALB/c mice were immunized either with whole cells or the cell wall extract. To immunize with whole cells, 0.5 ml of CS3 suspended in Ringer's solution to $\sim 10^8$ cells per milliliter ($OD_{660\ nm} = 1.0$) was injected intraperitoneally 3 times at 3 wk intervals and again 3 days before fusion. To immunize with the cell wall extract, 0.25 ml of extract (@ 40 μg of protein per milliliter) emulsified with an equal volume of Freund's incomplete adjuvant was injected subcutaneously 6 wk before fusion. A booster injection of 0.1 ml of extract without adjuvant was given intraperitoneally 3 days before fusion.

Myeloma cells P3/NS1/1-AG4-1 (NS-1) obtained from the American Type Culture collection were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM pyruvate and 2 mM L-glutamine and containing 10% fetal calf serum (FCS). They were used for fusion in exponential growth phase. Fusion of spleen and myeloma cells at a cell ratio of about 4:1 was done with 35 or 50% polyethylene glycol 1500 (BDH Chemicals Ltd., Poole, England) as the fusing agent (8). Fused cells from each fusion experiment were seeded directly into five 96-well plates in HAT selective medium (supplemented DMEM, 20% FCS, and hypoxanthine-aminopterin-thymidine selective agents) containing mouse thymocytes as feeder cells. After 7–10 days, wells with microscopically visible clones were screened for specific antibody production.

Hybridomas resulting from fusions using mice immunized with whole cells were screened against whole cells with an enzyme

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immunofiltration (EIF) procedure (10). Briefly, the EIF procedure involved depositing cells on nitrocellulose paper held in a 96-well Plexiglas manifold. Hybridoma culture fluid and enzyme immunoassay reagents were incubated on the top surface of the nitrocellulose and then washed with 0.04 M phosphate-buffered 0.8% saline (pH 7.2) (PBS) pulled through with vacuum. Protein A-peroxidase (Sigma) was used as the enzyme conjugate and *o*-phenylenediamine and hydrogen peroxide as 4 mg and 4 μl of a 30% solution, respectively, in 10 ml 0.1 M citrate-phosphate buffer pH 5 as substrate. Substrate conversion was estimated visually.

Hybridomas resulting from fusions using mice immunized with the cell wall extract were screened with an enzyme-linked immunosorbent assay (ELISA). This assay was carried out in 96-well polystyrene plates (Immulon 2; Dynatech Laboratories, Inc., VA) according to standard procedures (2). Antigens were loaded onto plates in 0.1 M carbonate buffer (pH 9.6) and reaction with monoclonal antibodies was monitored with Protein A-peroxidase (Sigma) or goat antimouse peroxidase (Kirkegaard and Perry Labs Inc., MD) conjugates and the *o*-phenylenediamine substrate as indicated above. Nonspecific adsorption of reagents to the polystyrene plates was blocked with 2% FCS plus 0.05% Tween-20 in antibody dilution buffers. All incubation steps were 1–2 hr and the level of substrate conversion was quantified by reading absorbance at 492 nm with a Titertek Multiskan MC plate reader.

Positive cultures were cloned and then subcloned one to three times in 96-well plates by limiting dilution. Hybridomas generated from single clones were stored in liquid nitrogen with dimethylsulfoxide as the cryoprotectant. Monoclonal antibodies from some of the hybridomas were produced from ascites by inoculating pristane-primed BALB/c mice intraperitoneally with hybridoma cells. Ascitic fluid was collected 10–15 days later and clarified by centrifugation. Immunoglobulins were purified by affinity chromatography on Protein A-Sepharose (13) or by gel filtration on Sephacryl S300 (5). They were adjusted to 0.5 mg protein per milliliter, and stored in 50-μl aliquots at −20 C.

Isotype determination. Isotypes of the monoclonal antibodies were determined by testing purified antibody in agar immunodiffusion against rabbit antisera specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (Litton Bionetics, Kensington, MD). Agar plates were prepared with 0.8% Difco purified agar, 0.85% NaCl and 200 ppm NaN_3. Each monoclonal antibody preparation was used to charge one of six 5-mm-diameter peripheral wells surrounding a center well that was charged with an antimouse immunoglobulin serum. The presence of precipitin bands was observed after incubation overnight at room temperature.

Immunofluorescence. The indirect immunofluorescence (IF) procedure was used as described previously (5,6). Four dilutions of each cell preparation to be tested were dried at 50 C on wells of multitest slides and heat fixed. Hybridoma culture fluid or purified monoclonal antibodies at 5 μg/ml in PBS plus 5% FCS and antimouse IgG conjugated to dichlorotriazinylaminofluorescein (Kirkegaard and Perry Labs) and diluted 1:50 in the same buffer were used as primary and secondary antibody reagents, respectively.

TABLE 1. Strains of *Corynebacterium sepedonicum* used for testing specificity of monoclonal antibodies

Strain	Obtained from	Isolated by	Geographic origin
CS3	R. J. Copeman	G. A. Nelson	Alberta
CX5 (ICPB)[a]	M. P. Starr	...[b]	New York
CS12	R. J. Copeman	H. Lawrence	New Brunswick
CS13	R. J. Copeman	H. Lawrence	New Brunswick
CS14	R. J. Copeman	D. Gross	Montana
CS15	R. J. Copeman	S. A. Slack	Wisconsin
CS16	R. J. Copeman	R. McKenzie	New Brunswick
CS17	R. J. Copeman	D. Hammon	Maine
CS20	R. J. Copeman	R. J. Copeman	British Columbia
CS106 (ICPB)	M. P. Starr	J. G. Leach	West Virginia
CS118 (ICPB)	R. J. Copeman	...	...
BRR7	R. J. Copeman	R. J. Copeman	British Columbia
P45 (ICPB)	R. J. Copeman	...	...
R1	...	S. H. De Boer	British Columbia
R2	...	S. H. De Boer	British Columbia
R3	...	S. H. De Boer	British Columbia
R4	...	S. H. De Boer	British Columbia
R5	...	S. H. De Boer	British Columbia
R6	...	S. H. De Boer	British Columbia

[a] ICPB strains originated from the International Collection of Plant Pathogenic Bacteria, Davis, CA.
[b] ... = Unknown.

RESULTS

The initial fusion experiments were carried out with splenocytes from mice immunized with whole cells. However, fusion frequency was low; only 5–10% of the wells developed hybridoma clones. Since results of screening for antibody production were erratic when whole cells were used in the standard ELISA procedure, the EIF procedure was used for screening against cells. Two stable hybridoma clones excreting antibodies to *C. sepedonicum* were produced from two separate fusion experiments by using whole cell antigens. These hybridomas were designated VRS-H-004-CS3-1 and VRS-H-005-CS3-2 (VRS-H-000 refers to Vancouver Research Station-hybridoma-file number). Monoclonal antibodies produced by them will be referred to as McAb 1 and McAb 2, respectively.

The lithium chloride cell extract was used subsequently in additional fusion experiments. PAGE analysis of the extract revealed the presence of multiple protein bands staining with Coomassie Blue and a single carbohydrate component staining with Schiff's reagent. Also, the extract reacted with McAb 1 and 2 in ELISA.

Two fusion experiments with splenocytes from mice immunized with the cell extract yielded a large number of hybridoma clones; almost all wells in the 96-well plates contained clones. Ten percent of these wells tested positive against cell extract in ELISA, and subsequently eight stable clones were obtained after recloning the first 25. Three of the clones, VRS-H-006-CS3-3, VRS-H-007-CS3-4, and VRS-H-008-CS3-5, that produced (respectively) McAb 3, 4, and 5 which reacted with whole cells in IF, were used for further study. An additional clone produced monoclonal antibodies with the same specificity pattern as McAb 3 and 4 and was therefore discarded.

Hybridoma culture fluid was used initially for testing specificity of the monoclonal antibodies by IF. Later, McAb 1, 2, 4, and 5 were purified and used for repeating the tests for specificity. McAb 1 and 2 were purified on a Protein A affinity column from which they eluted at pH 5.0 and 2.5, respectively. McAb 4 and 5 were purified by gel filtration since they did not react with Protein A. McAb 1 reacted in immunodiffusion with antiserum to IgG subclass 1 whereas McAb 2, 4, and 5 reacted with anti-IgM serum. Specificity in IF of monoclonal antibodies purified from ascitic fluid was consistently the same as from hybridoma culture fluid.

McAb 1–5 reacted with all of the 19 strains of *C. sepedonicum* listed in Table 1. Furthermore, McAb 2 and 5 did not react with any of the other plant pathogenic corynebacteria tested while McAb 1, 3, and 4 reacted with strains of *C. poinsettiae* and/or *C. fascians, C. insidiosum*, and *C. michiganense* pv. *iranicum* (Table 2). McAb 1, 2, 3, and 4 cross-reacted with one or more unidentified strains isolated from potato, but McAb 5 did not react with any of these strains (Table 3). The cross-reactions generally were weaker than reactions with *C. sepedonicum*.

DISCUSSION

Our results demonstrate the feasibility of producing monoclonal antibodies for serological detection of *C. sepedonicum*. Whole bacterial cell preparations were used successfully as immunogen to stimulate splenocytes, but the fusion experiment with splenocytes stimulated with the lithium chloride cell extract provided a greater number of clones. The cell extract also was simpler to use in ELISA than whole cells. Although the cell extract only was characterized

TABLE 2. Immunofluorescence reaction of monoclonal antibodies produced by five hybridoma cell lines with various plant pathogenic corynebacteria

Corynebacterium species	Strain	Monoclonal antibodies 1	2	3	4	5
C. sepedonicum	CS3[a]	++++[b]	++++	++++	++++	++++
	CS5[a]	++++	++++	++++	++++	++++
C. betae	CB101	−	−	−	−	−
	CB103	−	−	−	−	−
C. fascians	CF17	−	−	−	−	−
	CF107	±	−	+	+	−
C. flaccumfaciens	CF3	−	−	−	−	−
	CF8	−	−	−	−	−
C. insidiosum	C116	+	−	+++	+++	−
	C1102B	−	−	±	±	−
C. michiganense pv. *iranicum*	C1147	−	−	+	+	−
	C1148	−	−	−	−	−
C. michiganense pv. *michiganense*	CM1	−	−	−	−	−
	CM8	−	−	−	−	−
C. michiganense pv. *tritici*	CT102	−	−	−	−	−
	CT104	−	−	−	−	−
C. oortii	CO101	−	−	−	−	−
	CO102	−	−	−	−	−
C. poinsettiae	CP2	−	−	+++	+++	−
	CP109	−	−	−	−	−
C. rathayi	CR1	−	−	−	−	−
	CR101	−	−	−	−	−

[a] Reaction of only two strains of *C. sepedonicum* are shown here, but all the strains listed in Table 1 gave the same reaction.

[b] Symbols: ++++, bright fluorescence; +++, moderate fluorescence; ++, weak fluorescence; +, very weak fluorescence; ±, cells only partially stained, and with weak fluorescence; and −, no fluorescence.

TABLE 3. Immunofluorescence reaction of monoclonal antibodies produced by five hybridoma cell lines with unidentified, nonpathogenic bacteria isolated from potato

Bacterial strain	Monoclonal antibodies 1	2	3	4	5
A	++[a]	−	±	±	−
B	−	−	−	−	−
C	−	−	++++	++++	−
E	+	−	+++	+++	−
F	−	−	−	−	−
G	−	−	−	−	−
H	−	−	+++	+++	−
I	−	−	−	−	−
J	−	−	−	−	−
K	−	−	−	−	−
L	−	−	−	−	−
M	+	+	+++	+++	−
N	−	−	++++	++++	−

[a] Symbols: ++++, bright fluorescence; +++, moderate fluorescence; ++, weak fluorescence; +, very weak fluorescence; ±, cells only partially stained and with weak fluorescence; and −, no fluorescence.

as consisting of protein and carbohydrate components, apparently some of these constituents were somatic antigens. Monoclonal antibodies produced against whole cells reacted with the extract in ELISA and antibodies produced against the extract reacted with whole cells in IF.

The reaction of four different monoclonal antibody preparations with 19 strains of *C. sepedonicum* from at least eight different geographic areas indicates a high degree of conservation of some antigenic components of this bacterium. Since the specificities of McAb 3 and 4 were identical, these may be specific for the same epitope, but the specificity of the other monoclonals differed from one another. Our previous concern that monoclonal antibodies might exhibit too great a degree of strain specificity appears to be unfounded. Some of the monoclonal antibodies did react with *C. insidiosum, C. fascians,* and *C. michiganense* pv. *iranicum*—species with which our polyclonal antisera of *C. sepedonicum* also cross-reacted weakly. Two monoclonal antibody preparations cross-reacted with a strain of *C. poinsettiae* and this also has been observed previously with polyclonal antisera (12). These cross-reactions, in addition to the cross-reactions of four of five monoclonals with unidentified bacteria from potato, illustrate the difficulty in developing species-specific monoclonal antibodies. It is evident that certain antigenic determinants or epitopes are not only highly conserved on strains of *C. sepedonicum* but also may be present on other species.

McAb 5 was the most specific monoclonal for *C. sepedonicum*; it reacted with no other bacterium against which it was tested. Moreover, preliminary IF tests with McAb 5 of decayed, but ostensibly ring-rot-free potatoes did not reveal any positive cells. Nevertheless, more extensive testing of potato samples still needs to be carried out to ascertain whether there might be other as yet undetected cross-reacting bacteria associated with potato. It is often difficult to determine, however, whether a few fluorescing cells in a preparation are cross-reacting organisms or are a low population of *C. sepedonicum*. Often it is impossible to isolate bacteria that react in IF from preparations in which only a few IF-positive cells were observed. Our preliminary results indicate that staining with two different monoclonal antibody preparations conjugated separately with rhodamine and fluorescein dyes is possible and permits observation of two different antigenic determinants on the same bacterial cell. The probability of a cross-reacting cell having two antigens of *C. sepedonicum* is less than the probability of having one shared antigen.

Conway de Macario and Macario (3) stated correctly that ". . .it is already evident that monoclonal antibodies have set in motion a surge of far-reaching consequences in the area of bacterial identification and classification." We suggest that also in phytobacteriology there will be far-reaching consequences with the development of specific monoclonal antibodies for diagnosis of disease and detection of bacterial plant pathogens. Ring rot diagnosis, detection of symptomless ring rot infections, and further research into the ecology of *C. sepedonicum* will be enhanced with a specific monoclonal antibody probe.

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